Leiomyoma phosphoproteins involved in inhibition of oxidative stress and synthesis of reactive oxygen species

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Abstract. Uterine leiomyomas are benign smooth muscle cell tumors originating from the myometrium. The present study focused on leiomyoma and myometrium phosphoproteome enrichment by using immobilized metal affinity chromatography (IMAC). The phosphoproteome was analyzed by two-dimensional gel electrophoresis coupled with mass spectrometry. Western blotting was used for data validation. The results from IMAC identified 26 proteins significantly differentially phosphorylated in leiomyomas compared with normal myometrium. Three upregulated proteins (peroxiredoxin 2, protein disulfide isomerase family A member 3 and peroxiredoxin 4) were further validated by western blotting. Ingenuity pathway analysis revealed that four phosphoproteins were involved in the inhibition of oxidative stress and synthesis of reactive oxygen species. The present results demonstated for the first time an association between oxidative stress and phosphorylation in leiomyoma development.

Introduction

Uterine leiomyomas are benign smooth muscle cell tumors originating from the myometrium (1). During growth, a leiomyoma compresses the surrounding structures (the myometrium and connective tissue), causing the progressive formation of a sort of pseudocapsule, rich in collagen fibers (2), characterized by an abnormal extracellular matrix (ECM) and high interstitial fluid pressure (3). Several factors, such as growth factors, retinoic acid, ovarian hormones and vitamin D, are associated with tumor development (4,5).

Protein phosphorylation is a common post-translational modification (6) and usually occurs in serine, threonine or tyrosine residues (7). In uterine leiomyomas, altered protein phosphorylation is associated with the inhibition of apoptosis and the promotion of cell survival (8). Several receptors, such as estrogen receptor α (9) and receptor tyrosine kinases (10), exhibit increased phosphorylation levels in leiomyoma compared with the myometrium, subsequently promoting tumor growth. Other receptors, such as transforing growth factor β, mediate gene expression via the phosphorylation of Smad proteins (11).

Oxidative stress is associated with several gynecological disorders, such as uterine fibroids and endometriosis (12). Fletcher *et al* (13) reported that leiomyoma is characterized by an impaired antioxidant cellular system, suggesting a role of oxidative stress in its pathogenesis. In tumors, the increase in reactive oxygen species (ROS) is balanced by the upregulation of antioxidant systems (14). Pyruvate dehydrogenase kinase 1 is a ROS sensor activated by the rise of oxidative stress, leading to detoxification and cell survival (15). Oxidative stress stimulates phosphorylation of mixed-lineage protein kinase 3 by ERK1/2, enhancing the invasion of cancer cells (16). Identification of changes in protein phosphorylation levels in leiomyoma associated with oxidative stress may be useful for the understanding of the physiopathology of the tumor.

The present study used immobilized metal affinity chromatography (IMAC), two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) to analyze leiomyoma tissues. The aim was to identify differentially phosphorylated proteins involved in the suppression of oxidative stress and synthesis of ROS leading to tumor growth.

Materials and methods

Patients. Tissues samples were obtained from ten premenopausal patients who underwent hysterectomy for symptomatic uterine leiomyomas. All procedures conformed with the Declaration of Helsinki and were approved by

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the Review Board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy). All subjects involved signed a written informed consent. The median age of patients was 44 years, with a range of 36-48 years. The patients were recruited from January to Febuary 2019 at the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy), where all hysterectomies took place. Oncologic patients, HIV, HBV, HCV-seropositive patients, and patients with adenomyosis were excluded from the study. The patients had not received hormonal therapy in the three months prior to surgery.

Tissue samples. Two samples were collected from each patient: One from the central area of the leiomyoma and one from the unaffected myometrium. All leiomyomas were confirmed histologically as benign ordinary leiomyomas. Samples were stored at -80°C until proteomic analysis was performed.

Phosphoprotein isolation and 2-DE. One hundred mg of myometrium and leiomyoma tissue from ten patients were used for phosphoprotein isolation using the Phosphoprotein Enrichment kit (Thermo Fisher Scientific, Inc.). Tissues were homogenized in buffer [1% NP-40, 50 mM Tris-HCl (pH 8.0), NaCl 150 mM] with 1X Phosphatase Inhibitor Cocktail Set II (EMD Millipore), 2 mM PMSF and 1 mM benzamidine. The concentration of the supernatant was determined by Bradford assay. Tissue homogenates were then diluted to a final concetration of 0.5 mg/ml in lysis buffer provided by the Phosphoprotein Enrichment kit. Six ml of final samples were used for isolation of the phosphoproteins, according to the manufacturer's instructions.

For 2-DE analysis, a single patient-single gel strategy was adopted, and the analyzes were performed as previously described (17). For 2-DE analysis, 250 μg of proteins from each sample were denatured in 315 μl of dissolution buffer [7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 40 mM Tris, 65 mM dithiothreitol (DTT) and 0.24% Bio-Lyte 3/10 (Bio-Rad Laboratories, Inc.)] with a trace of bromophenol blue. ReadyStrip™ pH 4-7 18-cm immobilized pH gradient (IPG) strips (Bio-Rad Laboratories, Inc.) were rehydrated in dissolution buffer at 50 V for 12 h at 20°C, and isoelectric focusing (IEF) was performed in a PROTEAN IEF Cell (Bio-Rad Laboratories, Inc.). After the IEF, serial incubations were performed: first, the IPG strips were equilibrated for 15 min in an equilibration buffer [6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 30% glycerol and 1% DTT] and then in another equilibration buffer containing 4% iodoacetamide instead of DTT. For the second dimension, the equilibrated IPG strips were transferred to a 12% polyacrylamide gel.

After electrophoresis, gels were fixed in 40% methanol and 10% acetic acid for 1 h, and then stained for 16 h with SYPRO Ruby (Bio-Rad Laboratories, Inc.). 2-DE gels were scanned with a Molecular Imager PharosFX System (Bio-Rad Laboratories, Inc.). Double experimental replicates were performed per sample. For all gels, molecular weights were determined by comparison with Precision Plus Protein Prestained Standards (Bio-Rad Laboratories, Inc.) covering a range 10-250 kDa, and analyzed using the Proteomweaver 4.0 software (Bio-Rad Laboratories, Inc.).

Western blotting. Phosphoprotein extracts (20 μg) from IMAC columns used for 2-DE were separated by 12% SDS-PAGE and then transferred to a nitrocellulose membrane. The western blotting procedure for phosphoproteins was conducted as previously described (18). The membrane was blocked with 5% BSA in TBS/0.05% Tween-20 (TBST) for 2 h at room temperature. After BSA saturation, the membrane was incubated overnight at 4°C with primary rabbit polyclonal antibodies against peroxiredoxin 2 (PRDX2; 1:800; cat. no. SAB2101878), protein disulfide isomerase family A member 3 (PDIA3; 1:400; cat. no. SAB2107799) and peroxiredoxin 4 (PDRX4; 1:500; cat. no. SAB4301759; all Sigma-Aldrich; Merck KGaA). The membrane was washed three times in TBST for 10 min, and then incubated for 90 min at 4°C with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:3,000; cat. no. G4018; Sigma-Aldrich; Merck KGaA). Protein expression was visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Inc.), and the intensity of the signals was quantified by VersaDoc Imaging System (Bio-Rad Laboratories, Inc.). The intensities of the immunostained bands were normalized with the protein intensities measured with Red Ponceau (Bio-Rad, Laboratories, Inc.) from the same blot.

Trypsin digestion and MS analysis. Spots from 2-DE were digested and analyzed by MS, as described by Ura *et al* (19). After 2-DE gel excision, the spots were washed four times with 50 mM NH_4HCO_3 and acetonitrile (ACN; Sigma-Aldrich; Merck KGaA) alternatively, and dried under vacuum in a SpeedVac system. Three μl of 12.5 ng/μl sequencing grade modified trypsin (Promega Corporation) in 50 mM NH_4HCO_3 were added to gel spots for digestion (overnight at 37°C). Peptide extraction was achieved by three changes of 50% ACN/0.1% formic acid (FA; Fluka). Peptide mixtures were dried under vacuum and dissolved in 10 μl of 5% ACN/0.1% FA and 5 μl of each sample were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) on a 6520 Q-TOF mass spectrometer (Agilent Technologies, Inc.) coupled to a chip-based chromatographic interface.

Raw data files were converted into Mascot Generic Format (MGF) files with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies, Inc.) and searched with Mascot Search Engine (version 2.2.4; Matrix Science) through the Proteome Discoverer Software interface (version 1.4; Thermo Fisher Scientific, Inc.). Spectra were searched against the human section of the UniProt database (version July 2018, 95,057 sequences) using the following parameters: Enzyme specificity was set to trypsin with one missed cleavage allowed, while precursor and fragment ions tolerance were set to 20 ppm and 0.05 Da, respectively. Carbamidomethyl cysteine and oxidation of methionine were set as fixed modification and variable modification, respectively. MS/MS spectra containing <5 peaks or with a total ion count <50 were discarded. Proteins were considered as positive hits if, for each protein, at least two unique peptides were identified with high confidence (FDR <0.01%). For protein spots that did not return any significant hit, a Peptide Mass Fingerprint (PMF) was also performed with Mascot. All identified proteins were verified to have phosphorylated residues in PhosphoSitePlus database (www.phosphosite.org).

Table I. List of putative phosphoproteins with a significantly different abundance in leiomyoma compared with myometrium tissues.

Accession number	Spot number	Protein description	Gene symbol	Fold change	Protein class
A0A087WU08	7	Haptoglobin	HP	4.25	Hydrolase
P11021	17	78 kDa glucose-regulated protein	HSPA5	3.61	Chaperone
H9KV75	19	α-actinin 1	ACTN1	3.2	Cytoskeletal protein
A0A0C4DGB6	21	Albumin	ALB	3.1	Transfer/carrier protein
E9PFZ2	18	Ceruloplasmin	CP	3	Oxidoreductase
Q5JRR6	20	Ubiquitin-like modifier-activating enzyme 1	UBA1	3	Ligase
H7C3T4	3	Peroxiredoxin 4	PRDX4	3	Peroxidase
Q3BDU5	8	Prelamin A/C	LMNA	3	Cytoskeletal protein
H7BZ94	26	Protein disulfide-isomerase	P4HB	2.9	Oxidoreductase
P30101	11	Protein disulfide-isomerase family A member 3	PDIA3	2.75	Disulfide oxidoreductase
P10809	25	60 kDa heat shock protein	HSPD1	2.63	Chaperone
P18206-2	24	Isoform 1 of vinculin	VCL	2.54	Cytoskeletal protein
P02790	13	Hemopexin	HPX	2.45	Metalloprotease
P08238	16	Heat shock protein HSP 90β	HSP90AB1	2.32	Chaperone
P04792	3	Heat shock protein β1	HSPB1	2.3	Chaperone
E9PN50	9	26S protease regulatory subunit 6A	PSMC3	2.2	Hydrolase
Q8NBS9	10	Thioredoxin domain-containing protein 5	TXNDC5	2	Isomerase
P08603	22	Complement factor H	CFH	1.93	Complement control protein
P01023	23	α2-macroglobulin	A2M	1.9	Defence immunity protein
B7ZAR1	14	T-complex protein 1 subunit epsilon	CCT5	1.84	Chaperone
P28070	2	Proteasome subunit β type 4	PSMB4	1.8	Defence immunity protein
Q13409-2	15	Isoform 2B of Cytoplasmic dynein 1 intermediate chain 2	DYNC1H1	1.78	Cytoskeletal protein
P01023	19	Peroxiredoxin 2	PRDX2	1.7	Oxidoreductase
P01024	21	Complement C3	CO3	1.61	Complement control protein
Q16555	15	Dihydropyrimidinase-related protein 2	DPYSL2	1.5	Metalloprotease
P17661	5	Desmin	DES	0.07	Cytoskeletal protein

Fold-change was defined as the ratio of the mean % V according to the formula: %V = V single spot/V total spot of cases vs. controls (where V denotes the spot volume).

PANTHER and Ingenuity Pathway analyses. Phosphorylated proteins were analyzed by PANTHER 11.0 (Protein Analysis Through Evolutionary Relationships; http://www.pantherdb.org) and Gene Ontology (http://amigo.geneontology.org/rte). Proteins were then classified according to their protein class. Since the majority of the proteins identified were involved in multiple processes, only the most relevant ones were reported.

These proteins were analyzed by Ingenuity Pathway Analysis (IPA; Qiagen GmbH), as previously described (20). Selected genes were used to generate bio-functions. For the filter summary, only high confidence associations (predicted) or that had been experimentally observed were considered.

Statistical analysis. Statistical analyses were performed with the non-parametric Wilcoxon signed-rank test for matched samples
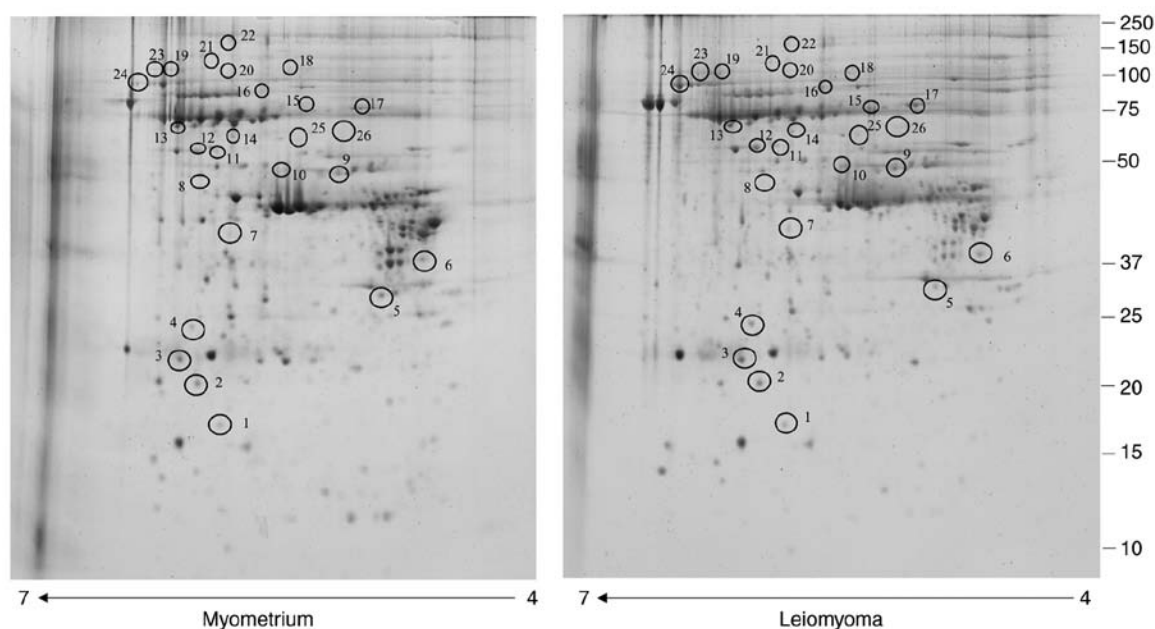
Figure 1. Two-dimensional electrophoresis map of normal myometrium and leiomyoma phosphoproteome enriched by immobilized metal affinity chromatography columns. Immobilized pH gradient 4-7 strips were used for the isoelectric focusing and 12% polyacrylamide gel was used for the second dimension. The numbered circles indicate the differentially phosphorylated spots.

for both 2-DE and western blot data. P<0.05 was considered to indicate a statistically significant difference. All analyses were conducted with Stata/IC 14.1 for Windows (StataCorp LLC).

Results

Enrichment by IMAC. For phosphoproteome enrichment, an IMAC column was used. This column, although non-specific, allowed us to efficiently enrich the phosphoproteome of both the leiomyoma and the myometrium tissues. An average of 1,800 spots were detected on gels for both types of the enriched phosphoproteome. The analysis revealed 26 protein spots (Table I) with a significantly different abundance (Fig. 1) in leiomyoma tissue compared with myometrium tissues. The correlation of gel-pairs performed well, with an average matching efficiency of ~80%. In the present study, only spots corresponding to putative phosphoproteins were considered, with the following criteria: fold change in %V (where V indicates the spot volume) ≥ 1.5 or ≤ 0.6 in intensity and P<0.05. Of these spots, 25 were significantly upregulated (>1.5-fold) while one was significantly downregulated (<0.6-fold).

Western blotting validation. Western blot analysis was used to confirm the alteration of three putative phosphorylated proteins: PRDX2, PDIA3 and PDRX4. The abundance of the propsphorylated forms of these proteins in five leiomyoma samples was compared to the matched normal myometrial tissue samples (same samples as used in 2-DE analysis) by western blotting (Fig. 2). The results demonsatrted that, in the five patients tested, phosphorylation levels of PRDX2, PDIA3 and PRDX4 were markedly higher in the leiomyoma tissues compared with the myometrium tissues, consistent with the 2-DE results.

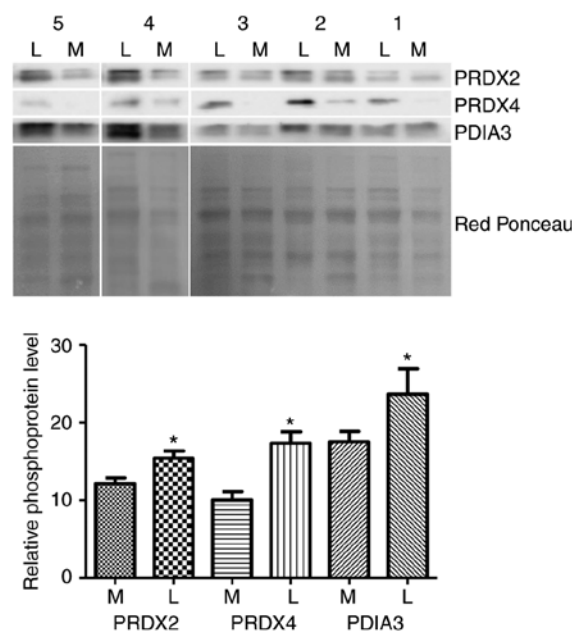*Bioinformatic analysis.* A PANTHER analysis of the identified proteins was conducted. Based on the PANTHER

Figure 2. Western blot analysis was utilized to confirm the alteration in the phosphorylation of the proteins PRDX2, PDRX4 and PDIA3 in paired myometrium and leiomyoma tissues from five patients. The intensity of immunostained bands was normalized against the total protein intensities measured from the same blot stained with Red Ponceau. The bar graph shows the relative average expression (band density) of PRDX2, PDRX4 and PDIA3 in the myometrium and the leiomyoma. Data are presented as mean ± standard deviation. *P<0.05. PRDX, peroxiredoxin; PDIA3, protein disulfide isomerase family A member 3; L, leiomyoma; M, myometrium.

classification system, the results revealed that these proteins could be grouped into: Chaperone, enzyme modulator, transfer/carrier protein, cytoskeletal protein, signaling molecule and in three enzyme classes, hydrolase, ligase and oxidoreductase.
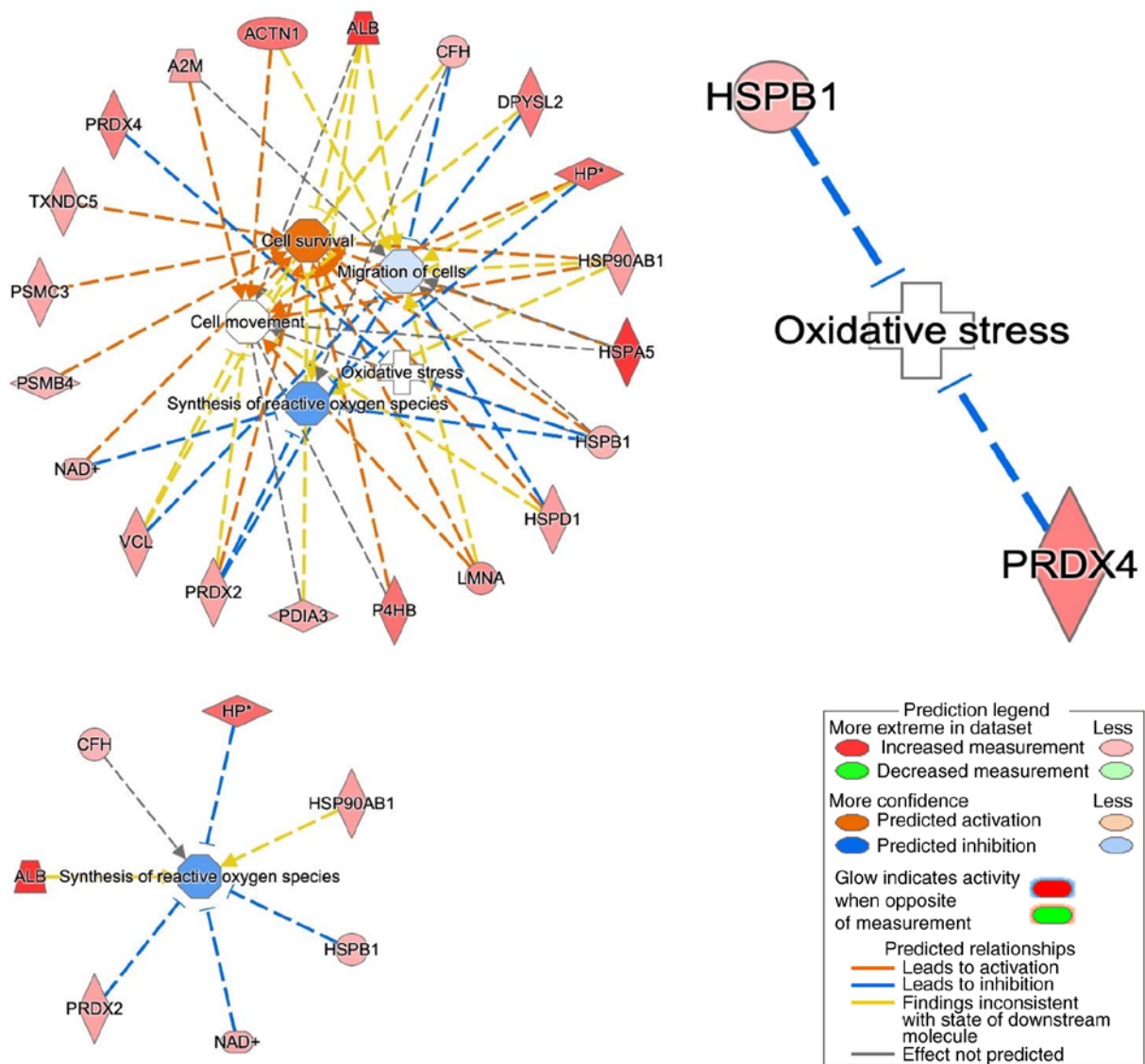
Figure 3. Network of significant biological functions involving the differentially phopshorylated proteins in leiomyoma. The functions of cell survival, migration of cells, cell movement, oxidative stress and synthesis of oxygen reactive species were found enriched by Ingenuity Pathway Analysis.

The *in silico* data analysis was then explanded by using the core analysis in the IPA software to construct a network in which these proteins were involved. The top networks in which these proteins were involved corresponded to: migration of cells, synthesis of ROS, cell movement, oxidative stress and cell proliferation of tumor cell lines (Fig. 3). Thirteen putative phosphoproteins were involved in the 'migration of cells' network, two were involved in the 'oxidative stress' network, five in the 'synthesis of ROS' network, 14 in the 'cell movement' network, while 13 were involved in the 'cell survival' network. Of these, four proteins [heat shock protein β1 (HSPB1), PRDX4, haptoglobin (HP) and PRDX2] suppress oxidative stress and synthesis of reactive oxygen.

Discussion

Phosphorylation of membrane proteins and kinases is fundamental for leiomyoma development (9,21). In the present study, by combining the IMAC column, 2-DE and MS, 26 putative phosphoproteins were identified to be differentially phosphorylated/expressed in leiomyoma tissues compared with myometrium tissues. By using western blotting, the differential levels of three putative phosphorylated proteins were confirmed: PRDX2, PDIA3 and PDRX4. All identified proteins have already been reported to be phosphorylated *in vivo* in the PhosphoSitePlus database, suggesting that the IMAC column was effective in isolating mainly phosphorylated proteins. Unfortunately, the validation experiments could not be performed using antibodies against the specific phosphorylated sites of these proteins, because these are not commercially available.

PRDX4 is a thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide. This enzyme protects against oxidative stress by detoxifying peroxides (22). The increase of this enzyme in tumors is associated with the protection of the cell from oxidative stress (23,24).

HSPB1 is a heat shock protein which maintains denatured proteins in a folding-competent state (25). Phosphorylation of HSPB1 inhibits apoptosis by protecting the cell from oxidative stress (26). The present study identified this putative protein

as differently phosphorylated in leiomyoma, suggesting a possible association of phosphorylated HSPB1 with the inhibition of oxidative stress and tumor growth.

PRDX2 is a thiol-specific peroxidase that serves a role in cell protection against oxidative stress by detoxifying peroxides, and as a sensor of hydrogen peroxide-mediated signaling events (27). This enzyme has an important role in cell survival by modulating the signaling involved in apoptosis and the phosphorylation of JNK, and by blocking the synthesis of ROS (28).

HP binds free hemoglobin (Hb), prevents oxidative stress and acts as a potent immunoreactive modulator in the acute phase (29). Fedorovych *et al* (30) found that the function of the HP/Hb complex in sera of patients with lung cancer was to neutralize superoxidative products (30). A similar mechanism may also be present in leiomyoma, in which the upregulation of phosphorylated HP may contribute to cell protection from reactive oxygen species.

In conclusion, the present study identified several putative phosphoproteins involved in oxidative stress in leiomyoma tissues. Further studies are needed to understand the role of phosphorylation in oxidative stress. The current data represented a step forward in the understanding of the mechanism involving oxidative stress in tumor growth.

Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to restrictions imposed by the Regional Bioethics Committee of Friuli-Venezia Giulia (Comitato Etico Unico Regionale), but can be available from the corresponding author prior to approval of the research protocol by the Review Board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy).

Authors' contributions

Conceived and designed the experiments: GR and FS. Performed the experiments: BU, GA, BG and DL. Analyzed the data: LM, BU and GA. Contributed to data analysis: GDL and FR. Wrote the paper: GDL, FR, BU, LM and GA. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Procedures incolving the use of human tissue were approved by the Review Board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy). All subjects involved signed a written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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